The Epstein-Barr Virus BcRF1 Gene Product Is a TBP-Like Protein with an Essential Role in Late Gene Expression

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That the expression of late genes is coupled to viral genome replication is well established for all herpesviruses, but the exact mechanisms of their regulation, especially by viral proteins, are poorly understood. Here, we report the identification of the Epstein-Barr virus (EBV) early protein BcRF1 as a viral factor crucial for the activation of late gene transcription following viral DNA replication during the productive cycle. In order to study the function of the BcRF1 protein, we constructed a recombinant EBV lacking this gene. In HEK293 cells, this recombinant virus underwent normal DNA replication during the productive cycle but failed to express high levels of late gene transcripts or proteins, resulting in a nonproductive infection. Interestingly, a TATT motif is present in the promoter of most EBV late genes, at the position of the TATA box. We show here that BcRF1 forms a complex with the TATT motif and that this interaction is required for activation of late viral gene expression. Moreover, our results suggest that BcRF1 acts via interaction with other viral proteins.

Epstein-Barr virus (EBV) is a human gammaherpesvirus that infects more than 95% of the human adult population. It is the causative agent of infectious mononucleosis (28) and is also associated with several B-cell and epithelial cell cancers, including Burkitt's lymphoma (BL), Hodgkin's disease, nasopharyngeal carcinoma, gastric carcinoma, and immunoblastic lymphoma in immunosuppressed individuals (52, 53, 54). After primary infection, EBV persists lifelong as a chronic asymptomatic infection by establishing latency in a subpopulation of resting memory B cells (4). Although the hallmark of latency is the absence of a complete viral productive cycle, EBV productively reactivates from latency in differentiated cells, and infectious virions are shed continuously into the saliva of healthy carriers (26) thereby allowing the efficient spread of the virus from host to host. Interestingly, there is strong evidence that both the latent and the productive forms of EBV infection are essential for the emergence of EBV-associated malignancies (33, 47).

EBV reactivation *in vivo* has been linked to the terminal differentiation of latently infected B cells into plasma cells (12, 23, 39, 41, 42). However, as yet, the signals and molecular mechanisms that trigger the switch from latency to the productive cycle *in vivo* remain unknown. In contrast, activation of the viral productive cycle *in vitro* has been well documented in cell lines established from EBV-positive BL biopsy specimens. The productive cycle can be induced by treating these infected cells with various agents: the tumor promoter 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) associated with sodium butyrate (BA) (60), anti-immunoglobulin (51), and tumor growth factor beta (TGF- β) (16). During the productive cycle, EBV genes are expressed in a highly ordered temporal cascade. Three different classes of viral genes have been characterized, namely, the immediate early (IE), early (E), and late (L) genes. The EBV IE genes BZLF1 and BRLF1 encode viral transcription factors called EB1 (or ZEBRA or Zta) and R (or Rta), respectively (9–11, 21, 27). Together, these factors induce the expression of the early genes. Many early genes encode proteins directly or indirectly involved in viral DNA replication, which is supported by OriLyt, the origin of replication used during the productive cycle of EBV. After viral DNA replication, most of the

late genes start to be transcribed. These late transcripts mainly encode structural proteins, ultimately leading to the assembly and release of infectious particles (reviewed in reference 35). While the regulation of immediate early and early gene expression in herpesviruses has been extensively studied, very little is known about the mechanisms regulating late gene expression. Immediate early and early gene promoters have the same structure as cellular gene promoters transcribed by RNA polymerase II. They typically consist of distal regulatory sequences upstream of the TATA box, which recruit TBP (TATA box-binding protein) at the origin of the formation of the preinitiation complex on the promoters. The structure of the late gene promoters appears to be less complex, but the number of late gene promoters that have been studied is not very large. Among the EBV late genes, only two late gene promoters have been studied in detail by testing deletion and point mutant forms (2, 7, 49). These studies showed that the TATA box, sequences around the transcription start site, and sequences up to about 30 nucleotides downstream of the transcription start site are the main determinants of late gene expression. This is also the case for other herpesvirus late gene promoters (25, 31, 32).

Although late gene transcription is tightly coupled to viral DNA replication, the underlying mechanism is poorly understood. Serio et al. (50) showed that activation of the BcLF1 and BFRF3 late gene promoters could be observed on transiently transfected reporter plasmids even if the reporter plasmids did not have the OriLyt sequence *in cis*. However, this reconstitution was dependent on EBV DNA replication occurring in the same cells (50). In contrast, Amon et al. (2) reconstituted a stable reporter system with the same late promoters, and they observed a specific

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dependence of late gene expression on OriLyt DNA replication in *cis*. The first set of data suggests that *trans*-acting factors produced after OriLyt-dependent DNA replication are required for late gene expression and that DNA replication increases the copy number of the viral episome, allowing titration of a putative repressor. The second data set also suggests that during or after DNA replication, the structure of the viral chromatin may change, allowing the transcription machinery increased accessibility to the late gene promoters.

Viral proteins implicated in the regulation of the viral late gene promoters have also not been well defined. In the case of herpes simplex virus 1 (HSV-1), several viral proteins (ICP4, ICP8, and ICP27) have been shown to be necessary for the efficient expression of late genes (19, 38, 40, 46). These factors interact with the general transcription machinery and could thus function to facilitate the assembly of the transcription preinitiation complex on the late promoters. However, since these viral proteins also play an important role in the regulation of the early promoters, the specific molecular mechanisms by which they control the retarded expression of the late genes is unknown. For other herpesviruses, like MHV-68 (mouse herpesvirus 68) and CMV (cytomegalovirus), several viral proteins have been shown to be necessary for the expression of late genes. MHV-68 encodes four proteins, ORF18, ORF24, ORF30, and ORF34, which are absolutely required for the expression of the late genes (3, 55, 56). Homologs of ORF18, ORF24, and ORF34 have been characterized in CMV (UL79, UL87, and UL95, respectively) and appear to have the same function (34, 45). Importantly, it should be noted that although a significant number of proteins are conserved among herpesviruses, these factors have no known homologs in HSV-1. Interestingly, EBV also encodes these three putative homologs (the genes encoding these factors are called BVLFI.5, BcRF1, and BGLF3, respectively), but no experimental assays have defined their precise function. However, from a genomewide fold recognition search, it has been proposed that the EBV BcRF1 gene encodes a protein with homology to TBP (57). This homology was also found in the BcRF1 homologs from various beta- and gamma-herpesviruses.

In the present study, we experimentally characterized the role of the BcRF1 protein during the EBV productive cycle. To define its function during virus replication, we engineered a recombinant EBV (EBV_{ΔBcRF1}) in which part of the BcRF1 open reading frame (ORF) was deleted. We found that cells harboring this recombinant virus are not capable of producing virions unless they are *trans*-complemented with a BcRF1 expression plasmid. We also found that BcRF1 is critical for the accumulation of late viral transcripts but not for viral DNA synthesis. Furthermore, we show that BcRF1 interacts specifically *in vitro* with the TATT sequence present on the promoters of the late genes. Moreover, BcRF1 mutants unable to interact with the TATT sequence are also unable to rescue viral production from EBV_{ΔBcRF1}. Our results show for the first time that EBV encodes a TBP-like protein that is absolutely required for viral late gene expression.

MATERIALS AND METHODS

Cell lines and transfections. HEK293 is a specific cell line originally derived from cultures of normal human embryonic kidney cells transformed with adenovirus type 5. Raji is a human EBV-positive Burkitt's lymphoma cell line. Mononuclear cells were purified from fresh blood buffy coats by density gradient centrifugation. CD19-positive B cells were isolated from

total lymphocytes using a human CD19 positive selection kit (magnetic beads were from StemCell). All cell lines were routinely grown in RPMI 1640 medium supplemented with 10% fetal calf serum (PAA). Transient transfections of HEK293 cells and derivatives were performed by electroporation (950 mF, 220 mV) using a Bio-Rad electroporator.

Recombinant plasmids. An EB1 expression plasmid was used for initiation of the EBV productive cycle (23). The entire BcRF1 ORF (B95.8 coordinates 125177 to 127429) was PCR amplified, cloned into the pCI expression plasmid (Invitrogen) under the control of a CMV promoter, and fused at its N terminus with the Flag epitope. BcRF1 mutants (BcRF1_{mutN1-N2} and BcRF1_{mutN8-N9}) were generated by using a QuikChange site-directed mutagenesis kit from Stratagene. In BcRF1_{mutN1-N2}, Asn420 and Asn422 were each replaced by an alanine using the oligonucleotide 5'-GGTTTCGCCTGCTCGTGTTCGCCACC GCTCGGGTGATCAACACTAAATTG-3' and its complement on the opposite strand. In BcRF1_{mutN8-N9}, Asn512 and Asn514 were each replaced by alanine using the oligonucleotide 5'-TTTATCCCGCGCCGT GTCAGCCTGGGCCTCGACTCTAGATCTCCATGGG-3' and its complement on the opposite strand. The pTATT-Luc reporter plasmid was created by cloning the following double-stranded oligonucleotide 5'-CCGG GTATTAAACCGGGTGGCAGCTCCTGGCAGTCATTCAC-3', which contains the -38 to -4 sequence from the BcLF1 minimum promoter, into the pGL2 vector (Promega). The pTATA-Luc reporter plasmid was generated by cloning a double-stranded oligonucleotide containing the same sequence as above, except that the TATT sequence was replaced by TATA (5'-CCGGGTATAAAACCGGGTGGCAGCTCCTGGCAGTCAT TCAC-3') in the pGL2 vector. Plasmids used for transfections were prepared by the alkaline lysis method and purified through an ion-exchange column (Macherey-Nagel).

Recombinant EBV plasmid. To generate a BcLF1 knockout (KO) mutant in the context of the EBV genome, we used the maxi-EBV technology described by Delecluse et al. (13). For this, we used the maxi-EBV plasmid (p2089; kindly provided by W. Hammerschmidt) that carries the prokaryotic F factor origin of DNA replication, the chloramphenicol resistance gene, and the gene for green fluorescent protein (GFP) under the control of the CMV promoter together with the hygromycin-resistant genes as a selectable marker in eukaryotic cells. The EBV BcRF1 KO mutant was constructed by replacing part of the BcRF1 ORF (B95.8 coordinates 125177 to 127429; accession number YP_401698.1) with the kanamycin (Kan) resistance gene using homologous recombination (17). To achieve this, the following composite primers were used: 5'-CGGCCAAGAGCG AAGACGAGGAGTCTGAGCGTCGAGACGCCCCCTCCACCCCGCTC GAAAAGTGGCCACCTGCAGAT-3' and 5'-GCAGCACTTGAATCTG ACTCCGATTGCGTAGCTCTAGCAGGGTAAAGAGCCGGTTTTTCAG GAACACTTAACGGCTGA-3'. The underlined sequences allowed PCR-mediated amplification of the Kan resistance gene. The rest of the primer sequences are homologous to BcRF1 sequences and were used for recombination of the amplified PCR product within the BcRF1 coding sequence. PCR amplification products were gel purified to remove traces of the parental plasmid and then introduced by electroporation (2,500 V) into *E. coli* DH10B cells carrying the BAC-EBV plasmid p2089 and the temperature-sensitive pKD46 helper plasmid encoding phage lambda red recombinase to foster homologous recombination. Cells were grown in Luria Broth (LB) with chloramphenicol (30 μg/ml) at 37°C for 1 h and then plated onto LB agar plates containing chloramphenicol (30 μg/ml) and kanamycin (25 μg/ml). Incubation at 42°C led to a progressive loss of the helper plasmid pKD46. After double selection, colonies were subsequently analyzed by restriction enzyme digestion to confirm correct recombination. One identified clone was selected, and its plasmid DNA was prepared using an ion-exchange column (Macherey-Nagel). The plasmid DNA was also analyzed by PCR amplification with primers located in regions of BcRF1 surrounding the introduction site of the Kan resistance cassette (5'-ATGACACAAGGTAAGAGGGAG-3' and 5'-CACTTGAGC ATCACGGCAG-3').

Plasmid rescue in *E. coli*. Circular plasmid DNA from HEK293_{ΔBcRF1} cells was extracted using a denaturation-renaturation method as previously described (20). *E. coli* strain DH10B was transformed with the viral recombinant DNA by electroporation, and clones were selected on LB plates containing chloramphenicol (30 μg/ml). Single bacterial colonies were amplified, and DNA plasmid preparations were subjected to digestion with BamHI and XhoI.

Stable clone selection. Recombinant EBV plasmid DNA (10 μg) was transfected into HEK293 cells using polyethylenimine reagent. At 1 day posttransfection, the cells were transferred to a cell culture dish (150-mm diameter), and hygromycin (100 μg/ml) was added to the culture medium for selection of stable HEK293 clones carrying the EBV recombinant plasmid. Outgrowing GFP-positive colonies were amplified for further investigation. The cell clone used in this study is referred to as HEK293_{ΔBcRF1}.

Induction of the virus productive cycle and infection of target cells. Producer cell clones HEK293_{EBV} carrying p2089 (kindly provided by W. Hammerschmidt) and HEK293_{ΔBcRF1} were transfected with an EB1 expression plasmid (1 μg/plate) to induce the viral productive cycle. In *trans*-complementation assays, HEK293_{ΔBcRF1} cells were cotransfected with pCI-Flag-BcRF1 plasmid (1 μg/well) or the empty plasmid. Three days posttransfection, cells were harvested for analysis of viral gene expression, and virus supernatants were filtered through a 0.45-μm Millipore filter unit and stored at 4°C. Viral titers were determined by infecting 2.5 × 10⁵ Raji cells with increasing dilutions of virus supernatants. Three days after infection, GFP-positive Raji cells were visualized using a fluorescence microscope and counted by fluorescence-activated cell sorting (FACS) analysis.

Electrophoretic mobility shift assay (EMSA). The ³²P labeled probe consisted of a double-stranded oligonucleotide containing the following sequence derived from the viral BcLF1 late gene promoter: 5'-GGACTCAGAATTATTAACCAGGGTGGCAG-3' (the TATT box sequence is underlined). Competition assays used the same but unlabeled oligonucleotide, the sequence derived from the viral BMLF1 early gene promoter (5'-CGCCTCCAGAATCATAAATAGGGCTGAGGC-3'), which is recognized by the TBP protein, or the irrelevant oligonucleotide ZRE-DR3 (5'-TATGCATGAGCCACAGGCATTGCTAATGTA-3') (24). BcRF1 protein was produced in a bacterial expression system as a glutathione S-transferase (GST) fusion protein as previously described (37). The protein was purified using glutathione Sepharose beads (GE Healthcare Life Science) according to the manufacturer's recommendations. GST-BcRF1 protein (2 μl) was incubated with 2 × 10⁴ cpm of labeled oligonucleotide. Incubations were carried out for 20 min at 4°C, in a volume of 20 μl containing 20 mM Tris-HCl (pH 7.9), 1 mM MgCl₂, 0.5 mM dithiothreitol (DTT), 10% glycerol, 100 mM KCl, and 0.1 μg poly(dI · dC). The GST-BcRF1-DNA complexes were separated from the noncomplexed DNA electrophoretically using a 4.5% polyacrylamide gel (migration, 10 V/cm) and were visualized by autoradiography.

Viral DNA replication analysis. EBV-infected cells were collected 72 h posttransfection, resuspended in lysis buffer (30) (200 mM NaCl, 20 mM Tris [pH 8.0], 50 mM EDTA, 0.2 mg/ml proteinase K, 1% sodium dodecyl sulfate [SDS]), and incubated at 55°C overnight. DNA was extracted with phenol-chloroform, treated with RNase A (100 μg/ml), extracted again with phenol-chloroform, precipitated with ethanol, and then resuspended in nuclease-free water. Viral DNA was quantified by semiquantitative PCR as described previously (5), using primers specific for the EBV early gene BMRF1 (5'-CGTGCCAAATCTTGAGGTTTT-3' and 5'-CACCCGGGGACTTTTATCTT-3').

Western blot analysis. Cells were resuspended in Laemmli buffer and lysed by sonication. The protein equivalent of 2 × 10⁶ cells was loaded into each lane of a 10% SDS-polyacrylamide gel and electroblotted onto a Hybond enhanced chemiluminescence (ECL) membrane (Amersham). Antibodies used for Western blotting were an anti-BZLF1 monoclonal antibody (Mab) Z125 (23), a rabbit polyclonal serum directed against the EB2 protein (22), an anti-gp350/220 Mab (clone OT6) (29), and an anti-

Flag M2 Mab (Sigma, F3165). After several washings in 0.1% Tween in Tris-buffered saline (TBS), blots were incubated for 1 h with anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (GE Healthcare Life Science), and immune complexes were visualized with the ECL reagent (Pierce).

RNA extraction and RT-PCR analysis. Transfected cells were washed twice with phosphate-buffered saline (PBS), and total RNA was extracted and purified using a NucleoSpin RNA kit (Macherey-Nagel). We performed reverse transcription-PCR (RT-PCR) on 0.5 μg of purified RNA. The primers used for RT-PCR were as follows: for BMRF1, 5'-CCAGACATACGGTCAGTCCATCTC-3' and 5'-TGCTTCACTTTCTTGGGGTG C-3'; for BDLF1, 5'-CACATGCATCTTGGCTCTGT-3' and 5'-CTATC TCATCCGGCACCATT-3'; and for BcLF1, 5'-GACAATTATCAAAAA CCACC-3' and 5'-GGTGCAGTTTGTAGTG-3'. Quantification of the RT-PCR results was done as previously described (1). As a control in our RT-PCR experiments, the primers 5'-GCTGCGTGTGGCTCCCGAGGA G-3' and 5'-ATCTTCATTGTGCTGGGTGCCAG-3' were used to amplify a 690-bp DNA fragment corresponding to endogenous β-actin mRNA.

Luciferase assays. Firefly luciferase activity from transfected cells was measured in a Veritas luminometer (Turner Biosystems) using a firefly luciferase assay system (Promega). Luciferase activity was measured for identical amounts of total protein, as evaluated by a Bradford assay.

RESULTS

BcRF1 is a DNA-binding protein interacting with the TATT box.

It is now well established that viral DNA regulatory elements are important in order to control the temporal cascade of EBV gene expression, and the presence of a TATT box in place of the canonical eukaryotic TATA box in the promoters of viral late genes appears to be very important for this regulation. From a genome-wide fold recognition search, Wyrwicz and Rychlewski (57) predicted that the EBV viral protein BcRF1 contains a domain with high structural homology to the archaeal TATA-binding protein TBP that could potentially recognize the TATT box of the late gene promoters. In order to determine whether BcRF1 was indeed a TATT-binding protein, we cloned the BcRF1 ORF in frame with the GST protein-coding sequence in a bacterial expression vector and produced the GST-BcRF1 fusion protein in a bacterial expression system. The purified protein was used in an electrophoretic mobility shift assay (EMSA) together with a double-stranded oligonucleotide probe corresponding to the region from -38 to -4 of the BcLF1 late promoter that contains a TATT box. As shown in Fig. 1, the GST protein alone did not form a complex with the TATT box probe (lane 1), whereas the GST-BcRF1 fusion protein did (lane 2). This complex was efficiently competed by an excess of unlabeled TATT box oligonucleotide (Fig. 1, lanes 3 to 6) as well as by an excess of unlabeled oligonucleotide originating from the early BMLF1 promoter and containing a canonical TATA box (lanes 7 to 10). However, the GST-BcRF1-TATT box complex was not competed with an excess of an irrelevant oligonucleotide (Fig. 1, lanes 11 to 14). These results demonstrate that the BcRF1 protein can bind to the TATT sequence. Similar to what has already been shown with the eukaryotic TBP protein (43), BcRF1 appears to be unable to discriminate between the TATA and TATT sequences, although BcRF1 seems to have a higher affinity for the TATT sequence.

Construction of a BAC-EBV recombinant clone carrying a deletion in the BcRF1 gene and propagation of recombinant virus. To study the function of BcRF1 in viral infection, we adopted a protein-based viral genetic approach. For this, we constructed a BAC-EBV recombinant clone, BAC-EBV_{ΔBcRF1}, in which we in-

FIG 1 The BcRF1 protein is a TATT-binding protein. BcRF1 protein was produced as a GST fusion protein in bacteria and used in a gel shift assay with the ³²P-labeled TATT_{BcLF1} probe. Competition was carried out with a 1-, 2.5-, 5-, or 10-fold excess of unlabeled TATT_{BcLF1} or TATA_{BMLF1} double-stranded oligonucleotide or with the unrelated ZRE-DR oligonucleotide. The GST-BcRF1–DNA complex is indicated by an asterisk.

roduced a large deletion in the BcRF1 open reading frame (ORF) (Fig. 2A). The BcRF1 early gene is located between the late genes BTRF1 and BcLF1. The BcRF1 open reading frame partly overlaps the BTRF1 ORF, these two genes being transcribed on the strand opposite to that containing the BcLF1 late gene. The ORF of the BcRF1 gene has been determined to be 2,252 bp, but its promoter region has not as yet been precisely mapped. To generate the ΔBcRF1 mutant, a 476-bp fragment from BcRF1 (B95.8 coordinates 126120 to 126596) was replaced with the kanamycin resistance gene sequence by homologous recombination in *E. coli* while the BcLF1 and BTRF1 ORFs and their promoters were left intact (Fig. 2A). The DNA of the resulting BAC-EBV_{ΔBcRF1} recombinant was analyzed by BamHI restriction enzyme digestion. As expected from the cloning strategy, the restriction profile confirmed that the 1,333-bp BamHI fragment that contains the BcRF1 gene had been replaced by a 1,955-bp fragment in the mutant (Fig. 2B). PCR amplification of the BcRF1 region of the BAC-EBV_{ΔBcRF1} genome with primers located on each side of the kanamycin cassette gave a fragment of 1,300 bp, as expected (Fig. 2C, lane 1). The fragment amplified from the original BAC-EBV genome, which does not contain the inserted kanamycin cassette, was smaller (950 bp) (Fig. 2C, lane 2).

BAC-EBV_{ΔBcRF1} DNA was prepared and transfected into HEK293 cells. Some of the GFP-positive cells that survived hygromycin selection were cloned, and several clones were amplified for further analysis. First, we screened these clones for permissivity to the EBV productive cycle. Cells were transfected with an EB1 expression plasmid in order to activate the productive cycle, and 2 days after transfection, expression of the viral early protein EB2 was analyzed by Western blotting. We then selected the cell clone with the highest expression level of the EB2 protein to perform further experiments. This clone is referred to as HEK293_{EBVΔBcRF1}. Next, we assessed the integrity of the viral genome present in this cell clone. To achieve this, viral episomes

FIG 2 Construction of recombinant BAC-EBV_{ΔBcRF1}. (A) Homologous recombination between the wild-type EBV genome (p2089) and a PCR fragment carrying the kanamycin resistance gene (Kana) flanked by 50 bp of BcRF1 sequences was performed in *E. coli* DH10B containing the pKD46 plasmid. Clones carrying the recombinant EBV were selected for chloramphenicol and kanamycin resistance. The resulting BAC-EBV_{ΔBcRF1} recombinant has the kanamycin gene inserted between positions 126120 and 126596 in the BcRF1 gene. The putative structural homology domain between the TATA-binding protein and BcRF1 is labeled TBP and was partially deleted in the recombinant. (B) Restriction enzyme analysis of the BAC-EBV_{ΔBcRF1} plasmid DNA. The BamHI restriction pattern of BAC-EBV_{ΔBcRF1} mutant DNA was compared to that of the wild-type BAC-EBV DNA. The modified DNA pattern is indicated by a plus sign and a minus sign. (C) PCR amplification analysis of the BAC-EBV_{ΔBcRF1} clone compared to the BAC-EBV clone carrying the wild-type EBV genome. The PCR was made with the two primers indicated by arrowheads in panel A. As expected, the PCR product is shifted due to the insertion-deletion in the BcRF1 gene.

were purified from the HEK293_{EBVΔBcRF1} clone and retransformed into *E. coli*. Restriction enzyme analysis of plasmid preparations obtained from bacterial clones demonstrated that the viral genome in the selected cell clone was complete (data not shown).

BcRF1 is essential for the production of infectious EBV particles. In order to test the production of progeny virus,

FIG 3 BcRF1 is necessary for virion production. (A) Raji cells were incubated with supernatants from (a) untransfected HEK293_{EBVΔBcRF1} cells, (b) HEK293_{EBVΔBcRF1} cells transfected with an expression plasmid encoding the EB1 protein in order to induce the productive cycle, and (c) HEK293_{EBVΔBcRF1} cells transfected with an expression plasmid encoding the EB1 protein and an expression plasmid encoding the BcRF1 protein in order to complement BcRF1 deficiency. GFP fluorescence of Raji cells was analyzed 48 h after infection. Corresponding images obtained with phase-contrast light microscopy were merged with the images showing UV fluorescence. (B) Immunoblot analysis of Flag-BcRF1, EB2, and EB1 proteins expressed in HEK293_{EBVΔBcRF1} cells that were either not transfected (lane 1), transfected with an EB1 expression plasmid (lane 2), or cotransfected with expression plasmids for both EB1 and BcRF1 (lane 3).

HEK293_{EBVΔBcRF1} cells were mock transfected or transfected with an expression plasmid encoding the EBV transcription factor EB1 to induce the productive cycle of EBV, either alone or together with an expression plasmid for the Flag-BcRF1 protein. Three days after transfection, cell supernatants were used to infect Raji cells. The presence of infectious EBV was visualized indirectly by GFP expression in infected Raji cells as described previously (13, 14, 22). At 3 days postinfection, no green Raji cells were detected when the cells were infected with supernatants from mock-transfected HEK293_{EBVΔBcRF1} cells (Fig. 3A, panel a) or from cells transfected with the EB1 expression vector alone (panel b), indicating that the HEK293_{EBVΔBcRF1} cells did not produce infectious viral particles. However, when the supernatants from HEK293_{EBVΔBcRF1} cells transfected with expression plasmids for both EB1 and the Flag-BcRF1 protein were used, a large number of green Raji cells were observed by UV light microscopy (Fig. 3A, panel c), indicating that ectopic expression of Flag-BcRF1 was able to rescue virus production from the HEK293_{EBVΔBcRF1} cells. This rescue of virus production was not due to an unusually high expression of the EB1 protein or proteins induced by EB1 such as the

FIG 4 BcRF1 is not required for viral DNA replication. Viral DNA replication in uninduced and induced (transfected with the EB1 expression plasmid) cells was assessed by semiquantitative PCR. Different dilutions of the purified DNA (50-fold to 5000-fold) were used for the PCR in order to demonstrate that the PCR is in a linear range. The PCR products were loaded onto a 2% agarose gel. The amount of viral DNA in the induced cells was compared to the amount of viral DNA present in the uninduced cells.

EB2 protein, since their expression levels were not altered by concomitant expression of Flag-BcRF1 (Fig. 3B). These results clearly demonstrate that the EBV BcRF1 gene is essential for the production of infectious EBV particles and that disruption of the BcRF1 gene can be functionally *trans*-complemented by transient expression of its product, the BcRF1 protein.

The viral early protein BcRF1 does not influence OriLyt-dependent DNA replication. We hypothesized that, as a putative late gene expression regulator, BcRF1 should not affect the lytic replication of the viral genome. In order to test this, HEK293_{EBVΔBcRF1} cells were either mock transfected or transfected with an expression plasmid for the EB1 protein so as to activate EBV's productive cycle, alone or together with a BcRF1 expression plasmid. At 48 h after transfection, the viral DNA was purified and quantified by PCR analysis. As a control, the HEK293_{EBV} cells were also used in order to compare the replication efficiency of the recombinant virus. The amount of viral DNA present in the untransfected cells served as a reference to determine the level of viral DNA replication in the EB1-induced cells. As shown in Fig. 4, induction of the productive cycle by transfection with the EB1 expression vector led to a 397-fold increase in the amount of viral DNA present in HEK293_{EBV} cells and a comparable 290-fold increase in the amount of viral DNA present in HEK293_{EBVΔBcRF1} cells. Coexpression of the BcRF1 protein in the HEK293_{EBVΔBcRF1} cells did not significantly change the amount of newly replicated viral DNA, demonstrating that the BcRF1 protein is not required for OriLyt-dependent viral DNA replication. As a control, we concomitantly determined the amount of virus produced in each condition and found, as before, that cotransfection of the cells with the BcRF1 expression plasmid leads to the production of infectious virus, demonstrating that the BcRF1 protein was functional in this experiment. Taken together, these results strongly suggest that despite being an early protein, BcRF1 does not participate in viral DNA replication.

FIG 5 BcrF1 enhances expression of the late viral genes. (A) Total RNA from HEK293_{EBV} or HEK293_{EBVΔBcrF1} cells that were either not transfected (lanes 1 and 5), transfected with an EB1 expression plasmid (lanes 2 and 6), or cotransfected with expression plasmids for both EB1 and BcrF1 (lanes 3 and 7) was purified, reverse transcribed, and analyzed by PCR using specific primers for actin (used to monitor the quality of mRNA purification and to normalize the amount of mRNA used under each condition), the BMRF1 early gene, and the BDLF1 and BcLF1 late genes. Lanes 4 and 8 show the results of a PCR amplification made with RNA used in lanes 3 and 7 in the absence of reverse transcriptase. (B) Immunoblot analysis of gp350/220, EB2, and EB1 proteins expressed from HEK293_{EBV} and HEK293_{EBVΔBcrF1} cells that were either not transfected (lanes 1 and 3), transfected with an EB1 expression plasmid (lanes 2 and 4), or cotransfected with expression plasmids for both EB1 and BcrF1 (lane 5).

The viral early protein BcrF1 is required for viral late gene expression. In order to demonstrate that BcrF1 protein is essential for the expression of viral late proteins, we used RT-PCR to analyze the expression of some early and late viral genes. Total mRNA from HEK293_{EBV} or HEK293_{EBVΔBcrF1} cells that were mock transfected, transfected with an EB1 expression vector, or transfected with both EB1 and BcrF1 expression vectors was extracted, reverse transcribed, and quantified by RT-PCR using primers specific for the early gene BMRF1 or the late genes BDLF1 and BcLF1. Cellular actin RNA was also quantified as an internal control for the amount of RNA and to show that there was no contaminating DNA in the RNA preparation. In mock-transfected HEK293_{EBV} or HEK293_{EBVΔBcrF1} cells, the early or late mRNAs were not (or were only very weakly) detectable by RT-PCR (Fig. 5A, lanes 1 and 5). As expected, in both HEK293_{EBV} and HEK293_{EBVΔBcrF1} cells transfected with an EB1 expression vector,

early BMRF1 mRNA was efficiently detected by RT-PCR (Fig. 5A, lanes 2 and 6), and the level of expression was not affected by coexpression of BcrF1 (Fig. 5A, lanes 3 and 7). The late BDLF1 and BcLF1 mRNAs were also efficiently expressed in HEK293_{EBV} cells transfected with the EB1 expression plasmid alone (Fig. 5A, lane 2), and their level was not modified when BcrF1 protein was coexpressed (Fig. 5A, lane 3). In contrast, the same late BDLF1 and BcLF1 mRNAs were only weakly detected in HEK293_{EBVΔBcrF1} cells transfected with the EB1 expression plasmid alone (Fig. 5A, lane 6), whereas they were clearly enhanced in cells cotransfected with the BcrF1 expression vector (Fig. 5A, lane 7). Taken together, these results demonstrate that BcrF1 is required for the induced expression of some viral late genes, probably through a direct transcriptional effect.

To substantiate our results obtained by RT-PCR, we also analyzed expression of the viral late gene gp350 by Western blotting in the HEK293_{EBVΔBcrF1} cells induced to the productive cycle in the presence or absence of the BcrF1 protein. The gp350 protein was not efficiently expressed in HEK293_{EBVΔBcrF1} cells when they were transfected with the EB1 expression plasmid alone (Fig. 5B, lanes 3 and 4), whereas it was expressed in HEK293_{EBV} cells under the same conditions (Fig. 5B, lanes 1 and 2). However, it was readily detected when the HEK293_{EBVΔBcrF1} cells were cotransfected with both the EB1 and BcrF1 expression vectors (Fig. 5B, lane 5). Expression of the early protein EB2 did not require the BcrF1 protein (Fig. 5B, lanes 4 and 5), again suggesting that the BcrF1 protein is required only for expression of late genes.

Binding of the BcrF1 protein to the TATT sequence is required for its function. We have shown that the BcrF1 protein is required for the expression of several late viral genes and that it interacts specifically with the TATT sequence known to be important for the transcriptional regulation of these genes. To determine whether DNA binding is important for the effect of BcrF1 on late gene expression, we introduced point mutations in the BcrF1 sequence in order to disrupt its binding activity, and we looked at the *trans*-complementation property of these mutants. The proposed structure of the BcrF1 DNA binding domain (57) suggests that the four asparagine residues at positions 420, 422, 512, and 514 in the BcrF1 protein are important for the recognition of the TATT sequence. Consequently, we mutated these two asparagine pairs to two alanine pairs by site-directed mutagenesis. These two mutant proteins were produced in bacteria as GST fusion proteins and used in a gel shift assay. As shown above, GST protein alone did not interact with the TATT probe, whereas the fusion protein GST-BcrF1 interacted efficiently (Fig. 6A, lanes 2 to 5). Interestingly, the two BcrF1 mutant proteins were unable to form a stable complex with the TATT probe even at a high concentration (Fig. 6A, lanes 6 to 9). These results support the prediction that BcrF1 interacts with DNA in a manner highly similar to that of the eukaryotic TATA-binding protein (TBP).

We then took advantage of these mutants to show that the capacity of BcrF1 to interact with the TATT sequence is essential for BcrF1 to rescue virus production. The HEK293_{EBVΔBcrF1} cells were cotransfected with the EB1 expression plasmid together with expression vectors for the BcrF1 or the BcrF1 mutant proteins. The supernatant from these cells was used to infect Raji cells, and after 3 days, the percentage of GFP-positive cells was evaluated by FACS analysis. The results presented in Fig. 6B show that BcrF1 protein efficiently rescued virus production from the BcrF1 deficient virus (compare lane 3 to lane 2), whereas the two BcrF1

FIG 6 Binding of BcRF1 to the TATT box is required for its function. (A) Characterization of BcRF1 mutant proteins that do not bind DNA. Two BcRF1 mutants in which Asn at positions 420 and 422 (BcRF1^{MutN1-N2}) or Asn at positions 512 and 514 (BcRF1^{MutN8-N9}) was mutated to Ala residues were produced as GST fusion proteins. Increasing amounts of the GST (lanes 2 and 3), GST-BcRF1 (lanes 4 and 5), GST-BcRF1^{MutN1-N2} (lanes 6 and 7), and GST-BcRF1^{MutN8-N9} (lanes 8 and 9) proteins were used in a gel shift assay with the ³²P-labeled TATT_{BcRF1} probe. The GST-BcRF1-DNA complexes are indicated with an asterisk. (B) Comparison of transcomplementation efficiency by BcRF1 and BcRF1^{MutN1-N2} or BcRF1^{MutN8-N9} proteins. Raji cells were infected with supernatant from HEK293_{EBVΔBcRF1} cells that had been either not transfected (lane 1), transfected with an EB1 expression plasmid (lane 2), or cotransfected with expression plasmids for both EB1 and BcRF1 (lane 3), BcRF1^{MutN1-N2} (lane 4), or BcRF1^{MutN8-N9} (lane 5). The amount of GFP-expressing Raji cells was determined by FACS analysis. The experiment was done three times, and the error bars represent standard deviations. (C) Immunoblot analysis for one representative experiment.

mutants completely failed to complement the deficiency (lanes 4 and 5). As shown by Western blot analysis (Fig. 6C), EB1 was well expressed in all transfected cells and the EBV productive cycle was efficiently induced, since the early protein EB2 was detected whenever EB1 was expressed (lanes 2 to 5). Furthermore, the two BcRF1 mutants were expressed at the same level as the original BcRF1

FIG 7 A TATT box element is necessary for the function of BcRF1. (A) Schematic representation of the luciferase reporter vectors used. (B) HEK293_{EBVΔBcRF1} cells were either transfected with the reporter vectors alone (columns 1 and 5), cotransfected with the reporter vectors and an EB1 expression plasmid (columns 2 and 6) or with a BcRF1 expression plasmid (columns 3 and 7), or cotransfected with expression plasmids for both EB1 and BcRF1 (lanes 4 and 8). The amount of luciferase expressed was measured 24 h after transfection. Luciferase activation was calculated relative to the value obtained with the pTATT-BcRF1-Luc reporter transfected alone. The experiment was done three times, and the error bars represent standard deviations. (C) Immunoblot analysis for one representative experiment.

protein (Fig. 6C, compare lanes 4 and 5 to lane 3). Taken together, these results demonstrate that the capacity of BcRF1 to interact with the TATT box of the late genes is absolutely required for the function of the protein.

BcRF1 protein activates expression of a reporter gene through the TATT box. In order to demonstrate that BcRF1 acts specifically on the TATT box of the late genes, we constructed a luciferase reporter plasmid in which the luciferase gene was under the control of a minimal promoter containing the TATT box and surrounding sequences from the BcRF1 late promoter (coordinates -38 to -4) or a mutated form of this promoter in which the TATT box was transformed into a TATA box (Fig. 7A). These reporter vectors were transfected into HEK293_{EBVΔBcRF1} cells with the EB1 expression plasmid required for activation of the viral productive cycle, alone or together with the BcRF1 expression vector. The luciferase activity was measured 24 h posttransfection. Expression of EB1 had a very weak effect on the luciferase activity expressed from pTATT-Luc (Fig. 7B, compare columns 1 and 2). Interestingly, expression of BcRF1 protein alone in these cells had no effect on the luciferase activity expressed from pTATT-Luc (Fig. 7B, column 3). However, when BcRF1 protein was coexpressed with the complete set of the viral proteins induced by EB1, we detected a high increase of luciferase activity from the TATT-Luc reporter (Fig. 7B, column 4). It is important to note that when

FIG 8 BcRF1 is also required for late gene expression in B cells (A) Primary B cells were immortalized with the recombinant Δ BcRF1 virus and then were either transfected with the reporter luciferase vectors alone as indicated (columns 1 and 5), cotransfected with the reporter vectors and with an EB1 expression plasmid (columns 2 and 6) or with a BcRF1 expression plasmid (columns 3 and 7), or cotransfected with expression plasmids for both EB1 and BcRF1 (columns 4 and 8). The amount of luciferase expressed was measured 24 h posttransfection. Luciferase activation was calculated relative to the value obtained with the pTATT-BcRF1-Luc reporter transfected alone. The experiment was done three times, and the error bars represent standard deviations. (B) Immunoblot analysis for one representative experiment.

we changed the TATT sequence to the canonical TATA sequence in our reporter gene, the basal expression level of the luciferase gene was greatly enhanced (Fig. 7B, compare column 5 to 1) and was not affected by the presence of the viral proteins (Fig. 7B, columns 6 to 8). Taken together, these results suggest that the TATT sequence inefficiently recruits TBP *in vivo* and that when BcRF1 is present, it increases the recruitment of a transcription machinery which is as efficient as that recruited by TBP. These results also clearly show that the BcRF1 protein alone does not induce transcriptional activity from the TATT box.

In order to show that the effect of BcRF1 on late gene expression was not restricted to epithelial cells and since EBV predominantly infects B cells, we generated a B cell line bearing the recombinant Δ BcRF1 virus. For this, purified primary B cells were infected with the recombinant virus produced from the HEK293_{EBV Δ BcRF1} cells. After 3 weeks, the number of outgrowing B cells was similar to that observed when the wild-type virus was used, demonstrating that the Δ BcRF1 virus was able to infect and transform primary B lymphocytes into lymphoblastoid cells (LCL) as efficiently as the wild-type virus. LCL _{Δ BcRF1} cells were then transfected with the luciferase reporter plasmids described above (Fig. 7A) and the EB1 expression plasmid required for activation of the viral productive cycle, alone or together with the BcRF1 expression vector. The luciferase activity was measured 24 h posttransfection. Similar to what was observed in the HEK293_{EBV Δ BcRF1} cells, EB1 expression had a weak effect on the luciferase activity expressed from pTATT-Luc (Fig. 8A, compare lanes 1 and 2), and expression of BcRF1 protein alone had no effect (Fig. 8A, lane 3). However, when the BcRF1 protein was coexpressed together with the complete set of the viral proteins induced by EB1, we observed a strong increase in luciferase activ-

ity with the TATT-Luc reporter (Fig. 8A, lane 4). In contrast to what was observed in the HEK293_{EBV Δ BcRF1} cells, when the TATT sequence was changed to a canonical TATA sequence in the reporter plasmid, the basal expression level was not significantly changed (Fig. 8A, compare lanes 1 and 5), and EB1 expression had a similar increasing effect on the luciferase activity from both reporters (Fig. 8A, compare lanes 2 and 7). However, in accordance with what was observed in the HEK293_{EBV Δ BcRF1} cells, overexpression of BcRF1 alone, or in the context of virus cycle reactivation (induced by ectopic expression of EB1), had no effect (Fig. 8A, compare lanes 6 to 8).

Hence, in both epithelial and B cells, the BcRF1 protein specifically requires the TATT sequence present on the core promoter of late genes and the presence of one or more other viral factors.

DISCUSSION

In this study, we demonstrated an essential function for BcRF1 in the EBV productive cycle, and we identified its role at a critical point when the virus progresses from viral DNA amplification to expression of the late genes. For this, we generated an EBV recombinant which expresses a mutated form of the early gene BcRF1. This mutant virus was used to analyze the function of the BcRF1 protein during the viral productive cycle. Without intact BcRF1, viral production is arrested after DNA synthesis, with a failure to efficiently express late gene transcripts. Our data thus demonstrate that late gene expression depends not only on viral DNA replication but also on the presence of the virally encoded factor BcRF1. Even if BcRF1 has been described as an early factor, it has no direct or indirect effect on viral DNA replication efficiency. This suggests that the viral transcription machinery required for expression of the late genes is formed early during the productive cycle. Since expression of the late genes also requires viral DNA replication (2), it is possible that the late transcription complex is recruited onto the late promoters during the DNA amplification step.

It has previously been shown that the EBV immediate early transcription factors EB1 and R are required, mainly in a cooperative manner, at all steps of the productive cycle program, although the relative contribution of the two immediate early proteins varies greatly with the target gene (18). Moreover, the EB2 protein is also required for the expression of the late transcripts. EB2 is a posttranscriptional factor required for the efficient cytoplasmic accumulation of several intronless viral mRNAs (5, 37, 48). For HSV-1, the immediate early viral proteins ICP4, ICP8, and ICP27 (the HSV-1 homolog of EB2) have been shown to be implicated in the regulation of the HSV-1 late genes (8, 19, 36, 38, 46, 58). By interacting with the general transcription machinery, they facilitate the assembly of preinitiation transcription complexes (6, 59). However, no specific proteins have yet been characterized for the expression of the late genes of this herpesvirus.

In contrast, homologs of BcRF1 are found not only in other gammaherpesviruses (e.g., ORF24 of KSHV and MHV-68) but also in betaherpesviruses (e.g., UL87 of CMV). In MHV-68, both viral DNA replication (15) and viral proteins ORF18 (3), ORF24 (55), ORF30, and ORF34 (56) have been shown to be required for late gene expression. In hCMV, three viral proteins, UL79 (45), UL87, and UL95 (34), have been shown to be required. UL79, UL87, and UL95 share sequence homologies with, respectively, ORF18, ORF24, and ORF34 from MHV-68 and BVL1, BcRF1, and BGLF3 from EBV. The CMV proteins are expressed as early

proteins, and they are recruited to prereplication complexes before the initiation of viral DNA replication (34), further supporting the hypothesis of an early recruitment of a specific complex onto the late promoters. However, the precise function of these proteins was unknown. We now show that BcRF1 from EBV is a TATA box-binding protein. *In vitro*, BcRF1 binds with almost the same affinity to a TATT sequence and a TATA sequence. However, *in vivo*, BcRF1 is able to activate transcription only from the TATT sequence driving the expression of the luciferase reporter gene. This suggests that in cells, either BcRF1 in a complex with other factors or has a large preference for the TATT sequence or the cellular TATA-binding protein (TBP) competes with BcRF1 for binding to the TATA sequence. It is interesting that the recombinant virus does present a weak expression of the late genes in the absence of BcRF1. We can speculate that this expression is the result of cellular TBP binding to the TATT sequences of the late promoters.

The specific sequence motif (TATT) of the core promoter of late genes (7, 49, 50, 57) is highly similar to the most common regulatory element (TATA box) of eukaryotic promoters. A consensus TATA box sequence for EBV early genes was defined as 5'-T-A-T-A-A>t-A-3', whereas a consensus TATT box sequence for EBV late genes was defined as 5'-T-A-T-T-T>a-A-3'. Although the difference between the two core elements is only one base pair, this appears to be sufficient to facilitate the recruitment of BcRF1 protein onto the late core element. The requirement of BcRF1 for late viral gene expression can be explained by the finding that interaction between TBP and the TATT sequence is less effective than binding to the consensus TATA sequence (44). Furthermore, the computational analysis of Wyrwicz and Rychlewski (57) that identified a structural homology between TBP from thermophilic archaeon *Pyrococcus woesei* and the central domain of BcRF1 reveals a difference in the amino acid composition of these two proteins in the 15 critical residues involved in the interaction of TBP with DNA. This could explain the preference of BcRF1 for the TATT sequence (57). The question of whether the TATT-binding protein BcRF1 has evolved from an ancestral TBP or represents a product of convergent evolution is as yet unresolved. We speculate that the BcRF1 protein acts in cooperation with other viral factors, possibly with the products of the BGLF3 and BVLFI genes, whose functions are not yet known but whose MHV-68 homologs have been found to be necessary for MHV-68 late transcription. TBP in the TFIID complex interacts with different TBP-associated factor (TAF) proteins, and it is known that the composition of the TAFs present in the complex could determine the specificity of the induced transcription. In parallel with this, we can speculate that BcRF1 could also interact with one or several cellular TAFs that could modulate the selectivity of BcRF1 to the late viral promoters. How the transcription complex responsible for the induction of late gene transcription is formed and recruited onto the late promoters is under investigation.

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